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TITLE: Regulation of C-myc Gene Expression by Potassium Channel Blocker Quindine in MCF-7 Human Breast Cancer Cell Line

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13. ABSTRACT ***(Maximum 200 Words)***

C-myc is a protooncogene, that plays an important role in regulation of cell cycle progression, cellular differentiation and apoptosis. Its abnormal expression was reported in 32% of breast cancers and tumorigenic properties of c-myc overexpression was confirmed by both *in vitro* and *in vivo* models of breast cancer. Hence, it is important to understand the precise molecular mechanisms of c-myc regulation. The goal of this proposal is to elucidate how depolarization of the membrane potential in response to potassium channel blocking agent quinidine regulates expression of c-myc gene. Specific aim #1 was to test effects of quinidine on c-myc promoter activity in transient reporter gene assay. We mapped 614 bp minimal region of c-myc promoter, that is sufficient to confer responsiveness to quinidine. Fine mapping of this region using PCR and characterization of the nature of DNA/protein(s) interaction in that region are our next goals. Specific aim #2 was to test effects of quinidine on c-myc transcription rate using nuclear run-on assay. This aim remained unchanged and will be completed within the proposed time schedule. The significance of this proposal is that its results will improve our understanding of c-myc gene regulation and might discover new targets for breast cancer therapy.

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Table of Contents

Introduction

C-myc is a protooncogene, which means that it can cause tumor formation when its proper regulation is disrupted. C-myc gene product has a regulatory role in cell cycle progression, cellular differentiation, and apoptosis or programmed cell death (1,2). Abnormal expression of the c-myc was reported in 32% of breast cancers (3) and tumorigenic properties on c-myc overexpression in mammary tissue have been confirmed by both *in vitro* and *in vivo* models of breast cancer. Despite on extensive studies in this area, the precise regulatory mechanisms of c-myc gene expression are still poorly understood. Previous research in our laboratory showed, that changes in cell membrane potential due to potassium channel blockade play an important regulatory role in cell cycle progression and proliferation in MCF-7 human breast cancer cells. Our preliminary results showed that quinidine, potassium channel blocker, suppressed estradiol-stimulated c-myc mRNA levels in MCF-7 cells, which is paralleled by G1/G0 arrest and cellular differentiation. The goal of this proposal is to understand the molecular mechanisms by which depolarization of the membrane potential subsequent to potassium channel blockade regulates activity of c-myc gene. Aim # 1 was to test the hypothesis that depolarization of the membrane potential by quinidine suppresses c-myc promoter activity in a transient reporter gene assay, using c-myc promoter region linked to the luciferase reporter gene. If there is a suppression at this level, then a series of 5'-deletion mutants of c-myc promoter will be constructed to map a minimal region of c-myc promoter that is responsive to the changes in membrane potential. Aim # 2 was to test hypothesis that depolarization of the membrane potential by quinidine regulates the rate of c-myc transcription, using nuclear run-on assay with appropriate 5'-probe (c-myc exon 1) and 3'-probe (c-myc exon 3) to distinguish effects of quinidine on transcription initiation and elongation respectively. The results from this study will improve our understanding of the c-myc gene regulation and may discover new targets for breast cancer therapy.

Body of the Report

Annual Summary.

The goal of this proposal is to understand the molecular mechanisms by which depolarization of the membrane potential subsequent to potassium channel blockade regulates activity of c-myc gene expression. Specific aim #1 consisted of two parts: 1A was to test the hypothesis that depolarization of the membrane potential by potassium channel blocker, quinidine, suppresses c-myc promoter activity in a transient reporter gene assay using c-myc promoter region linked to the luciferase reporter gene; 1B was to construct a series of 5'-deletion mutants of c-myc promoter to map a minimal region of c-myc promoter that confers responsiveness to quinidine.

Aim 1A: we have found that quinidine significantly suppresses estradiol- stimulated c-myc promoter activity in MCF-7 cells in a dose-dependent manner, as shown in figure 1. Quinidine did not have any effects on the activity of purified luciferase protein. That was an important control, since in a reporter gene system, activity of the promoter region is determined indirectly by measuring luciferase activity. We also shown, that luciferase activity of promoterless plasmid was 0.01% of that in plasmid containing c-myc promoter. To assay the specificity of quinidine's effect on c-myc promoter, we conducted the same experiments with Cyclin D1 promoter-luciferase construct. We found no significant changes in cyclin D1 promoter activity in response to quinidine. Quinidine's effect on c-myc promoter will also be confirmed using a single c-myc promoter-CAT construct kindly provided by Michael Moore, Marshall University, WV.

Aim 1B: five different 5'-deletion mutants of c-myc promoter linked to the luciferase reporter gene were kindly provided by Bert Vogelstein and Ken Kinzler, Johns Hopkins University, Baltimore. These constructs were used to determine a minimal promoter region that confers responsiveness to quinidine. Schematic structures of these constructs are shown in figure 2A. The restriction sites are shown on a top of the figure. Negative numbers represent the position of corresponding restriction site in respect to transcription start site (+1) for c-myc P1 promoter. Figure 2B shows the effects of quinidine, estradiol or quinidine+estradiol (Q+E) on luciferase activity in MCF-7 cells transiently transfected with corresponding mutant construct. Quinidine significantly suppressed both basal and estradiol-stimulated c-myc promoter activity to about 25-35 % in each mutant construct. The smallest region on c-myc promoter, sufficient to confer quinidine responsiveness, Quinidine Responsive Element (QRE), was mapped to 614 bp region (Frag-E) spanning from –100 to +514 in respect to +1 start site for P1 promoter of c-myc gene. This region contains several different responsive elements, including a half site for estrogen responsive element (ERE), Sp1/Sp3 binding site, cAMP-responsive element (CREB), serum responsive element (SRE), and Ets and E2F transcription factor binding sites. All these elements may be involved in mediating quinidine's response on c-myc promoter. Fine mapping of QRE will be performed by making small promoter deletions using polymerase chain reaction (PCR) and targeted single-stranded DNA primers. Also, restriction endonuclease junction sites in Frag-D will be sequenced to check for the mutations in estrogen responsive element, which might explain a lack of estradiol induction of c-myc promoter in this construct.

PI decided to add part C to the aim #1, that would include characterization of the nature of protein(s)-DNA interaction in fine mapped QRE using electrophoretic mobility shift assay (EMSA). EMSA has been widely used to assay for protein-DNA interactions. It is based on the retarded mobility of protein-DNA complexes during polyacrylamide gel electrophoresis. Briefly,

crude nuclear extracts containing many DNA binding proteins are incubated with the radiolabeled DNA sequence of interest (probe) in the reaction mix. This reaction mix then loaded to polyacrylamide gel and separation of the proteins and DNA is allowed by electrophoresis. Free probe migrates faster than protein-probe complexes. Formation of retarded protein-DNA complexes is analyzed by autoradiography. Protein-DNA interaction is time and concentration-dependent. Longer incubation time and high protein concentrations may result in nonspecific protein-DNA interactions, which do not occur naturally. That is why it is very important to use specificity control, such as the ability of excess "cold" DNA fragment to compete with proteins for binding to the probe. EMSA protocol optimized in Dr. Evans (Department of Biochemistry, WVU) laboratory will be adapted for use with a fluorescently labeled DNA probe.

Experimental design and methods for aim #1C: MCF-7 cells arrested in G1/G0 by DMEM medium containing 2% dextran/charcoal treated FBS will be treated with: 1) no drug, 2) 2nM estradiol, 3) 90 uM quinidine, 4) quinidine+estradiol. One hour later nuclear extracts will be isolated and incubated with fluorescently tagged QRE for 30 min at 4°C in the reaction buffer. Cold QRE sequence will be added to duplicate samples prior to the addition of nuclear extracts. Protein(s)/DNA complexes will be separated on 6% nondenaturing polyacrylamide gel. The gels will be visualized on the Phosphorimager using ImageQuant software.

The results of this experiment depend on the nature of protein or protein complexes interacting with QRE. If these proteins are transcription repressors, then quinidine will promote binding of these proteins to the QRE to suppress c-myc transcription. In contrast, if these proteins are transcription activators, then quinidine will suppress binding of these proteins to the QRE. In order to identify which proteins are involved in the protein-QRE interaction, the nuclear extracts will be pretreated with antibodies to different transcription factors whose binding sites are present in QRE, such as Sp1, Sp3, CREB, E2F before incubation with the probe. If protein, the antibodies to which were used is present in the protein-DNA complex, then pretreatment of the nuclear extracts with its antibodies will produce supershift on a gel.

If QRE sequence will reveal no homology with any known DNA consensus sequence from the GeneBank, and if we will fail to identify the nature of QRE-binding proteins by the supershift method, then purification and cloning of this new protein or proteins that are responsible for the regulation of c-myc gene expression in response to changes in the membrane potential in MCF-7 cells will be our long-term goal. This novel protein will represent potential target for the development of new anticancer drugs.

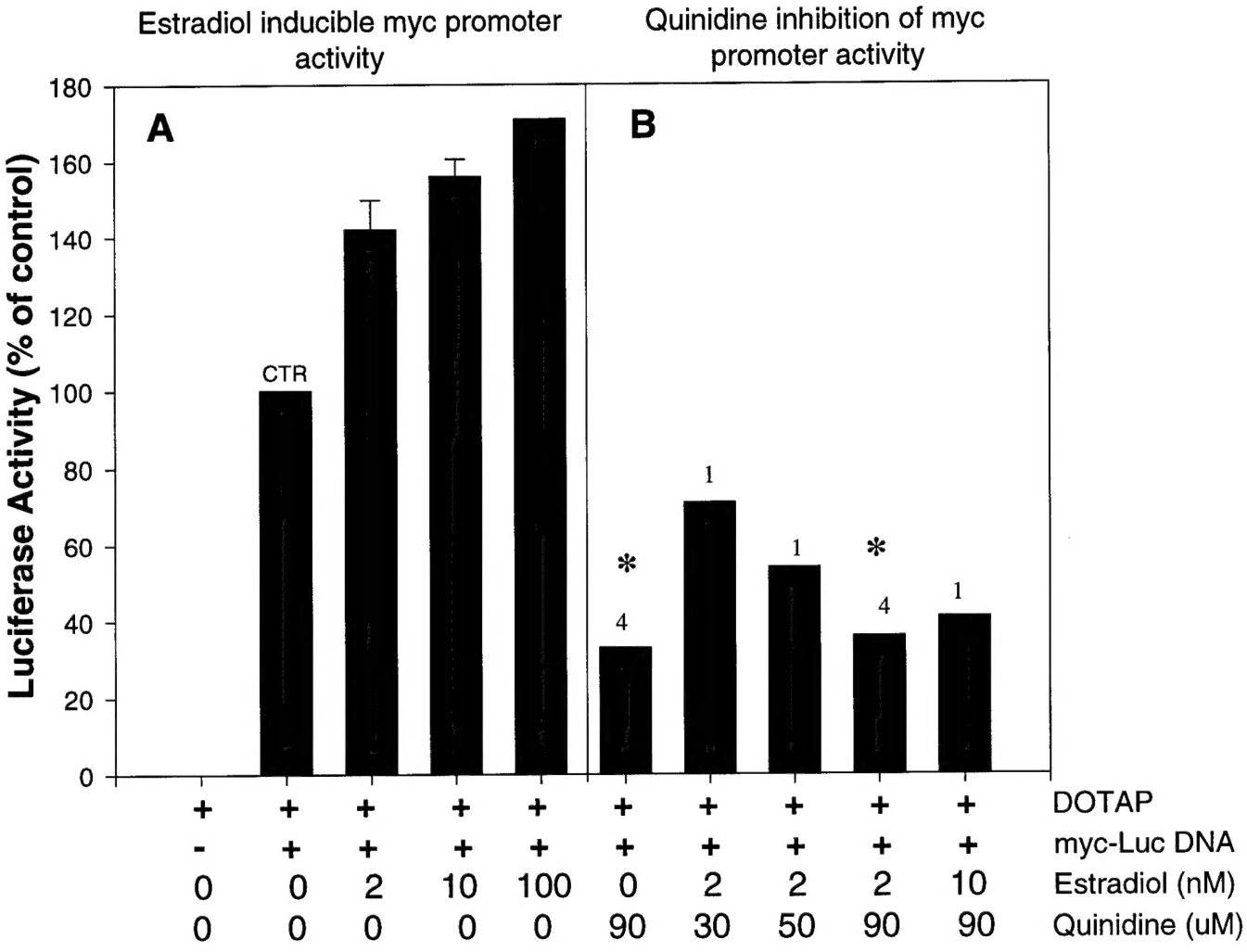
Aim #2 was to test hypothesis that depolarization of the membrane potential by quinidine regulates the rate of c-myc transcription using nuclear run-on assay with appropriate 5'-probe (c-myc exon 1) and 3'-probe (c-myc exon 3) to distinguish effects of quinidine on transcription initiation and elongation respectively. This aim remained unchanged and will be completed within proposed time frame.

Training and Research Accomplishments.

During the period of June,1999 - June,2000 I successfully completed PhD course work requirements in Pharmacology and Toxicology Department with a cumulative GPA of 3.88. I have also successfully passed the qualifying examinations, consisting of the writing and defending the NIH format grant proposal, describing my research project and an oral examination, that tests the general knowledge acquired from the course work material. I presented one research seminar and

did two journal club presentations on current scientific literature, as well as actively participated in the teaching assignments for the Medical Pharmacology course. I also presented my research data in the Van Liere Research Convocation in West Virginia University, where I was ranked fifth out of 26 participants within School of Medicine. I attended the American Society for Cell Biology (ASCB) meeting in December, 1999 in Washington, DC and the American Association for Cancer Research (AACR) meetings in April, 1999 in Philadelphia and in April, 2000 in San Francisco, where I presented two abstracts. I was also a second author in two other AACR abstracts and one manuscript recently submitted to the Journal of Biological Chemistry. At the end of this fiscal year I received Swiger Award for the best graduate students from the Pharmacology/Toxicology department for my academic and research accomplishments.

Figure 1. Transient transfection assay of human c-myc promoter regulation in MCF-7 cells



MCF-7cells were transiently transfected with 2.8 kb c-myc promoter-luciferase vector using DOTAP transfection reagent. After termination of the transfection cells were pretreated for 20 min with indicated concentrations of Quinidine, then 2 or 10 nM Estradiol was added and 24 hrs later cells were harvested for the luciferase assay. DOTAP luciferase activity = .01% of control. All Luciferase Units were calculated per 200 ug of cell extract proteins and then expressed as a % of control value (CTR). Bar graph represents mean +/- SEM of 3 independent experiments, unless otherwise shown by the number on a top of the bars.

Quinidine did not have significant effects on the activity of cyclin D1 promoter tested under the same conditions (n =4). In these experiments luciferase activity in control cells was set to 100%, luciferase activity in quinidine treated cells = 120 % +/- 15.3 (SEM)

Figure 2A

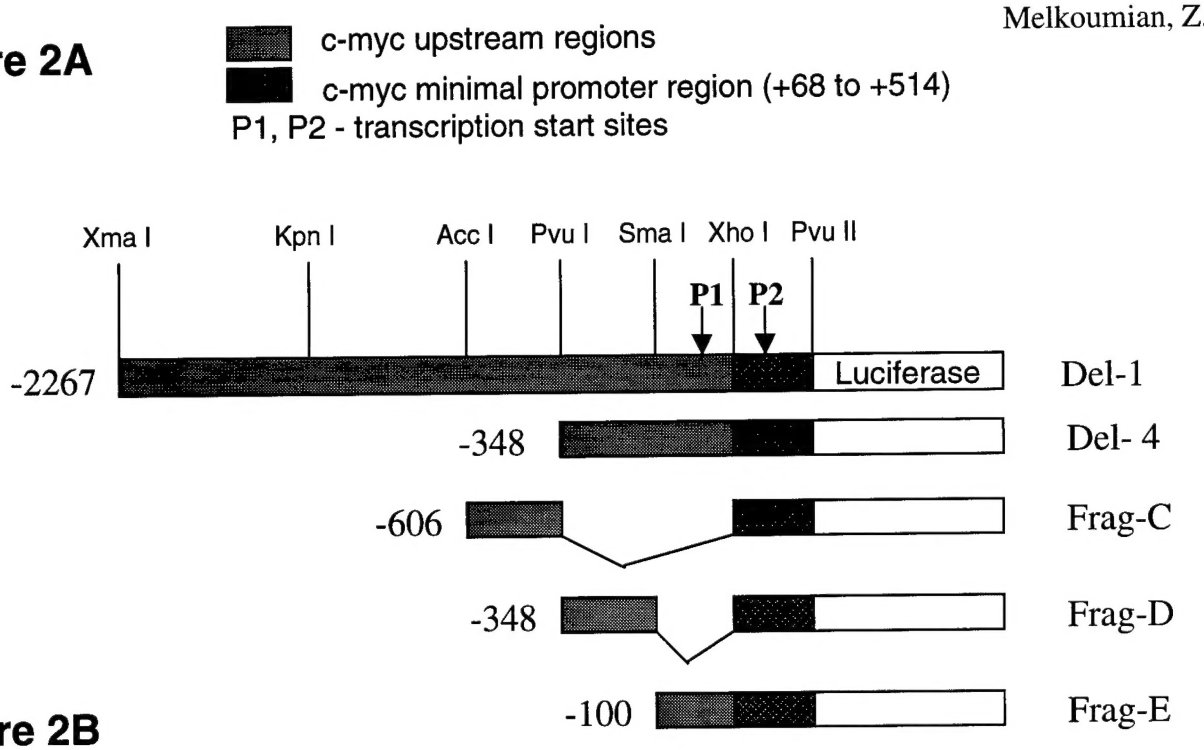
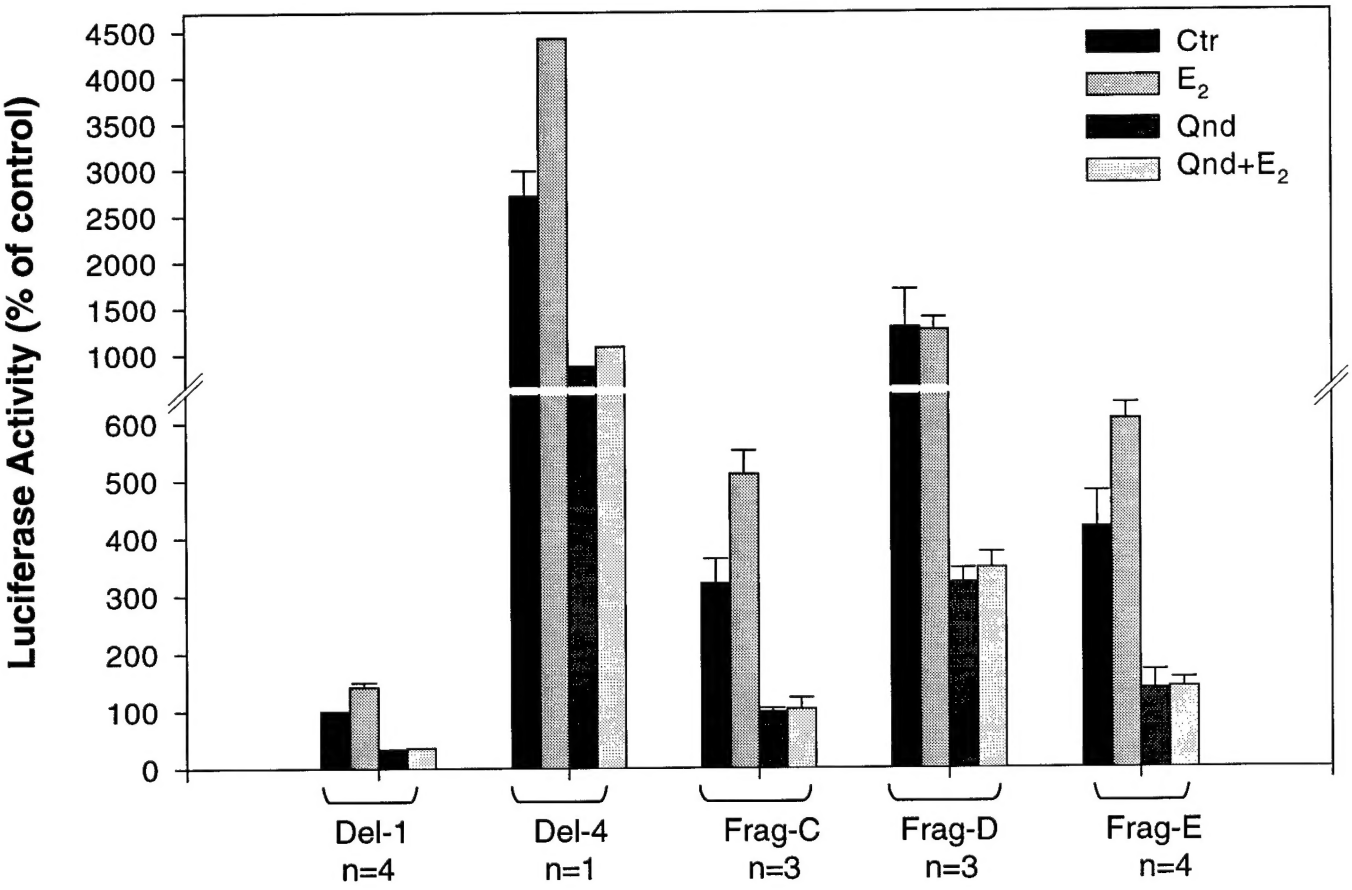


Figure 2B



Luciferase activity of control cells in each mutant construct is expressed as a % of luciferase activity in Del-1 control cells, which is set as 100% (absolute value was at least 1500 light units). Luciferace activity of the treated cells in each construct is expressed as a percent of activity in the corresponding control cells. n = number of experiments. The values of bars represent mean +/- SEM of all experiments.

Key research accomplishments during April, 1999- May, 2000:

- Progressing on specific aim # 1A and 1B of the US Army proposal
- Writing and defending of the NIH grant proposal on my research project
- Presentation of the seminar on my research project in the Pharmacology and Toxicology department, WVU
- Presentation of my research data in the Van Liere Research Convocation in West Virginia University
- Two journal club presentations of current scientific literature
- Receiving of the Swiger Award for the best graduate students from the Pharmacology/Toxicology department, WVU
- Publication and presentation of two abstracts on the national meetings (first author)
- Two other abstracts presented on the national meetings (second author)
- One manuscript submitted to the Journal of Biological Chemistry (second author)

Reportable outcomes:

1. Suppression of c-myc mRNA levels and G0/G1 cell cycle arrest of MCF-7 and MCF-7ras human breast cancer cells in response to quinidine. **Melkoumian, Z.K.**, Strobl, J.S. Proc. American Association for Cancer Research, 1999. Abstract # 188.

2. Quinidine suppresses c-myc promoter activity and induces differentiation of MCF-7 human breast cancer cells. **Melkoumian, Z.K.**, Strobl, J.S. Molecular Biology of the Cell, 1999. Abstract # 2483.

3. Quinidine activates p21 $^{WAF-1/CIP-1}$ expression and phosphorylation of pRb prior to onset of apoptosis in MCF-7 human breast cancer cells. Zhou,Q., **Melkoumian, Z.K.** Proc. American Association for Cancer Research, 1999. Abstract # 369.

4. Differentiation of human breast tumor cells by quinolines. Johnson, D. N., **Melkoumian, Z.K.**, Lucktong, A., Strobl, J.S. Molecular Targets and Cancer Therapeutics, AACR-NCI-EORTC, Washington, DC, Novermber, 1999. Abstract # 437.

5. Rapid induction of histone hyperacetylation and cellular differentiation in human breast tumor cell lines following degradation of histone deacetylase-1. Qun Zhou, **Melkoumian, Z.K.**, Lucktong, A., Moniwa, M., Davie, J.R., Strobl, J.S. Journal of Biological Chemistry (in revisions).

Conclusions:

During the first funded year (1999-2000) PI made significant progress on the specific aim #1A and 1B, as has been proposed in the original grant. The minimal region on the c-myc promoter, which is sufficient to confer responsiveness to quinidine, Quinidine Response Element (QRE), was identified. QRE was mapped to 614 bp region spanning from –100 to +514 in respect to start site for P1 promoter. Further fine mapping of QRE will be performed by making small promoter deletions using PCR and targeted single-stranded DNA primers. Aim #1C was added

by PI. This aim includes characterization of the nature of protein(s)/DNA interactions in QRE using a new gel mobility shift assay with fluorescently labeled probe. Completion of this aim is very important as a next logical step and might discover new targets for breast cancer therapy. Specific aim # 2 remained unchanged and will be completed according to the originally proposed time schedule.

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1. Amati, b. and Land, H. Myc-Max-Mad: a transcription factor network controlling cell cycle progression, differentiation and death. Current Opinion in Genetics and Development. 4:102-108, 1994.

2. Henriksson, M. and Luscher, B. Proteins of the Myc network: essential regulators of cell growth and differentiation. Advances in Cancer Research. 68: 109-182, 1996.

3. Liu, E., Santos, G., Lee, W.M., Osborne, K,. Benz, C.C. Effects of c-myc overexpression on the growth of MCF-7 human breast cancer cells. Oncogene. 4: 979-984, 1989.

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Suppression of c-myc mRNA levels and G0/G1 cell cycle arrest of MCF-7 and MCF-7ras human breast cancer cells in response to quinidine. Melkoumian, Z.K., Strobl, J.S. *Department of Pharmacology and Toxicology, West Virginia University, Morgantown, WV 26506.*

Quinidine (Q), a potassium channel blocking agent, causes cell cycle arrest of MCF-7 cells in G1 phase by 30 hrs with subsequent accumulation of cells in G0 by 48 hrs. After 72 hrs, Q led to cell death through apoptosis. To clarify the molecular mechanisms involved in these responses we examined effects of Q on c-myc mRNA levels by Northern blot analysis. C-myc is a proto-oncogene whose transient expression is required for cell cycle progression in the early G1 phase. Q caused concentration-dependent suppression of c-myc mRNA levels in MCF-7 cells which correlated with concentration-dependent arrest of cells in G1 phase. MCF-7 cells stably transformed with Ha-ras oncogene (MCF-7ras) showed a 3.5-fold increase in c-myc mRNA abundance compared with MCF-7 cells, which correlated with 3.5-fold acceleration of the G1 transit time. Q reduced c-myc mRNA expression and increased both the G1 cell fraction and cell doubling time of MCF-7ras cells. In contrast to MCF-7 cells, MCF-7ras cells resisted entry into G0 and failed to undergo apoptosis in response to Q. Mitochondrial swelling and cell death by necrosis occurred with MCF-7ras cells. We conclude Q inhibits c-myc mRNA abundance, cell cycle progression and is able to suppress ras-stimulated growth.
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Quinidine activates p21$^{WAF-1/CIP-1}$ expression and phosphorylation of pRb prior to onset of apoptosis in MCF-7 human breast cancer cells.

Zhou, Q., Melkoumian, Z.K., Strobl, J.S. *Department of Pharmacology and Toxicology, R.C. Byrd Health Sciences Center, West Virginia University, Morgantown, WV 26506, U.S.A*

Our previous work demonstrated that quinidine(Q) caused depolarization of the plasma membrane in MCF-7 cells by blocking potassium channels. Cell cycle analysis indicated that Q inhibition of MCF-7 cell growth occurred through induction of G1 arrest at 30 hr with a concomitant reduction in the proportion of cells in S and G2+M phases .Cells entered G0 phase following 48 hr exposure to drug (90uM) and underwent apoptotic cell death after 72 hr in Q. Q increased p21 expression by 12 hr , possibly contributing to the arrest of cells in G1 phase. Protein levels of p21 were increased to a maximum of 8.0- fold at 36 hrs, and remained 3.0 -fold higher than in control cells at 72 hrs. Q increased the levels of hypophosphorylated pRb 3.0-fold by 12hr, and stimulated expression of the phosphorylated form of ppRb beginning at 24 hr. Elevated levels of ppRb persisted in Q-treated cells through the 72 hour time point. CyclinD1 protein levels also increased after 24 hr. In conclusion, Q induces a transition of cells from G1 arrest into G0 that is followed by apoptosis, and this process is associated with the activation of conflicting signaling pathways evident as increased levels of the cyclin -dependent kinase inhibitor,p21, increased cyclin D1, and increased levels of ppRb .

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Quinidine suppresses c-myc promoter activity and induces differentiation of MCF-7 human breast cancer cells.

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Our previous results showed that quinidine causes cell cycle arrest in early G1 phase and inhibition of proliferation in MCF-7 human breast cancer cells by 30 hours of treatment. After 48 hours in quinidine MCF-7 cells begin to express several differentiation markers: accumulation of cytoplasmic lipid droplets, loss of Ki-67 expression, and exhibit an increase in the cytoplasmic/nuclear volume ratio. To understand the underlying mechanism of these changes we studied the effects of quinidine on expression of c-myc, an early-response gene, involved in the regulation of cell cycle progression, cell proliferation and differentiatiation. We found that 90 uM quinidine almost completely down-regulated Myc protein by 90 min of treatment and kept Myc suppressed during a 24 hour observation period. C-myc promoter activity in response to quinidine was tested using the 1.8 kb human c-myc promoter region linked to the luciferase reporter gene in a transient expression assay. Our results revealed that quinidine suppressed both basal and estradiol-stimulated c-myc promoter activity by 24 hours. We conclude that quinidine down-regulates c-myc gene expression and protein levels within 24 hours and prior to the appearance of a more differentiated phenotype in MCF-7 cells.

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Abstract #

Differentiation of human breast tumor cell lines by quinolines.

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Our goal is the design of novel compounds that cause differentiation of human breast tumor cells. Based upon evidence that quinidine caused differentiation of MCF-7, MCF-7*ras*, MDA-MB-231, and MDA-MB-435 human breast tumor cell lines, we screened a series of quinolines and other antimalarials for induction of a more differentiated phenotype of MCF-7 cells. Oil Red O (ORO) is a histochemical stain that detects accumulated lipid droplets, a marker of differentiation in breast epithelial cells. Normal human mammary epithelial cells (HMEC) are 97% ORO-positive, however, less than 3% of control MCF-7 cells are ORO-positive. The differentiating agent, retinoic acid (10 μM), was used as the positive control. The most active agents tested were quinine (maximum effective concentration, 90 μM), quinidine (90 μM) and primaquine (10 μM), and these caused positive ORO staining in 79-89 % of MCF-7 cells by 72 hours. Quinoline (50 μM) and chloroquine (10 μM) were less active, and 20-23 % of cells were ORO-positive by 72 hours. Quinolinic acid (50 μM), mefloquine (1 μM) and halofantrine (1 μM) were inactive as differentiating agents (<8 % ORO-positive). All agents tested, except quinoline and quinolinic acid, caused apoptosis of MCF-7 cells at concentrations greater than those which elicited a maximum ORO response. Our results demonstrate that a subset of quinoline antimalarial agents cause differentiation and apoptosis in human breast tumor cell lines. Primaquine was the most potent differentiating agent identified, and may be of use as a lead compound in the design of breast tumor differentiating agents. Support: WVU School of Medicine, USARMC, BC981114, and Edwin C. Spurlock Cancer Research Fellowship.

Abstract classification
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- ☐ Agents with unknown/uncertain targets
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- ☐ Biological response modifiers and cytokines
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Rapid Induction of Histone Hyperacetylation and Cellular Differentiation in Human Breast Tumor Cell Lines Following Degradation of Histone Deacetylase-1

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Abbreviations

DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; HDAC, histone deacetylase; MG-132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; PBS, phosphate buffered saline, 140 mM NaCl-2 mM KCl - 8 mM $Na_2HPO_4 \cdot 7H_2O$ – 1.5 mM KH_2PO_4, pH 7.0; Rb, retinoblastoma protein; SAHA, superoylanilide hydroxamic acid.

Summary

Quinidine is a natural product that exhibits pharmacological activity as an anti-malarial and an anti-arrhythmic agent. In a series of studies using the MCF-7 cell line as a model, we showed that quinidine is also an anti-proliferative agent in human breast tumor epithelial cells. Quinidine caused the arrest of MCF-7 cells in G1 phase of the cell cycle followed by a G1 to G0 transition that was complete by 48 h. Cell death ensued via apoptosis between 72 and 120 h. The present experiments demonstrated that MCF-7 cells undergo cellular differentiation under the influence of quinidine as evidenced by the accumulation of lipid droplets and the reorganization of the cytokeratin 18 cytoskeleton. Four additional cell lines derived from human breast epithelial cells (MCF-7*ras*, T47D, MDA-MB-231, MDA-MB-435) also showed growth arrest and differentiation in response to quinidine. In all cases, the differentiation response was more marked in response to quinidine than to retinoic acid. Quinidine was not overtly cytotoxic to normal human mammary epithelial cells (HMEC). Hyperacetylated histone H4 appeared within 2 h of the addition of quinidine to the medium, and levels were maximal by 24 h. Analysis of the cell cycle regulatory proteins in response to quinidine performed in MCF-7 cells showed elevated p21/WAF1, hypophosphorylation and suppression of retinoblastoma protein (pRb), and down-regulation of cyclin D1. This was the same profile as reported in cells that were induced to differentiate by trichostatin A and trapoxin which act as direct inhibitors of histone deacetylase (HDAC) activity. Quinidine did not show evidence for direct inhibition of

HDAC enzymatic activity *in vitro.* HDAC1 protein was undetectable in MCF-7 cells 30 min after addition of quinidine to the growth medium. The proteasome inhibitor MG132 completely protected HDAC1 from the action of quinidine. We conclude that quinidine is a novel differentiating agent that causes loss of HDAC1 protein levels via a proteasomal-sensitive mechanism.

Introduction

Histone deacetylase (HDAC) proteins comprise a family of related proteins that act in conjunction with histone acetyltransferase proteins to modulate chromatin structure and transcriptional activity via changes in the acetylation status of histones. Histones H3 and H4 are the principal histone targets of HDAC enzymatic activity, and these histones undergo acetylation at lysine residues at multiple sites within the histone tails extending from the histone octamer of the nucleosome core. The association of HDAC proteins with mSin3, N-CoR or SMRT and other transcriptional repressors has led to the hypothesis that HDAC proteins function as transcriptional co-repressors [reviewed in 1]. The spectrum of genes that show alterations in gene transcription rates in response to decreased HDAC activity is quite restricted [2]. Yet, small molecule inhibitors of the enzyme histone deacetylase (HDAC) such as trichostatin A (TSA), superoylanilide hydroxamic acid (SAHA), trapoxin, and phenylbutyrate cause major alterations in cellular activity including the induction of cellular differentiation and apoptosis [3-5]. Trichostatin A, SAHA and trapoxin stimulate histone acetylation by acting as direct inhibitors of HDAC enzyme activity [6]. Trichostatin A, SAHA and trapoxin possess lysine–like side-chains and act as chemical analogs of lysine substrates. Molecular models based upon the X-ray crystal structure of an HDAC-like protein indicate that trichostatin A and SAHA can bind within the active site of the HDAC enzyme and interact with a zinc metal ion within the catalytic pocket that is critical for enzymatic activity [7]. Trapoxin is an irreversible HDAC enzyme inhibitor [8].

Much remains to be learned about the biochemical events subsequent to HDAC inhibition that lead to cell cycle arrest, cellular differentiation, and apoptosis. However, a spectrum of biological responses characteristic of HDAC inhibitors has emerged, including cell cycle arrest in G1, elevated p21/WAF1 expression, hypophosphorylation of retinoblastoma protein (pRb), hyperacetylation of histones, particularly H3 and H4, and apoptosis. Histone hyperacetylation is directly linked to the activation of p21 transcription and is p53-independent [5]. This observation provides an important link between HDAC inhibition and cell cycle arrest because p21/WAF1 plays a critical role in causing G1 cell cycle arrest via inhibition of the G1 cyclin-dependent kinase family [9]. Overexpression of p21/WAF1 has also been associated with apoptosis, but the mechanism of p21/WAF1-induction of apoptosis requires further investigation [10].

Cancer therapy that targets the activity of genes or gene products controlling cell cycle progression, differentiation and apoptosis is a promising new strategy. Because HDAC inhibitors regulate the cell cycle and cause both cellular differentiation and apoptosis, they comprise an interesting group of compounds with potential for development into a new category of clinically significant anti-tumor agents. Single, key protein targets for "gene-regulatory chemotherapy" are difficult to identify due to the existence of parallel, functionally overlapping signaling cascades. For this reason, use of cancer therapies that target multiple intracellular signaling pathways, such as observed with the HDAC inhibitors, is an intriguing approach that addresses the problem of redundancy in growth signaling pathways. In this regard, the HDAC inhibitor phenylbutyrate was recently shown to have clinical anti-tumor activity [11].

Quinidine is a natural product therapeutic agent originally used as an anti-malarial and as an anti-arrhythmic agent. Previous studies with human breast tumor cell lines demonstrated that quinidine (90 uM) is an anti-proliferative agent as well. Quinidine arrested cells in early G1 phase, and induced apoptosis by 72-96 hr in MCF-7 cells [12], but the biochemical basis for the anti-proliferative effect of

quinidine was not well understood. To clarify the molecular mechanisms of the anti-proliferative activity of quinidine, we investigated the effects of quinidine on histone acetylation and cell cycle regulatory proteins. In this report, we show that quinidine causes hyperacetylation of histone H4, downregulation of HDAC1 protein levels, and cellular differentiation in a panel of human breast tumor cell lines. We conclude that quindine is a novel differentiating agent that stimulates histone hyperacetylation as a result of HDAC1 protein degradation.

Methods

Cell culture

Permanent cell lines derived from patients with breast carcinomas were used in these studies. MCF-7 cells, passage numbers 40 - 55, MCF-7-ras [13], T47D, MDA-MB-231, and MDA-MD-435 cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone Laboratories, Inc., Logan, Utah), 2mM glutamine, and 40 ug/ml gentamicin. Experiments were performed in this medium supplemented with 5% FBS. The cells were maintained at 37 C in a humidified atmosphere of 93% air/7% CO_2. After six days, cells became about 70-80% confluent and were passaged at a 1:5 ratio (MCF-7) or at a 1:10 ratio (all others). Normal human mammary epithelial cells (HMEC) were obtained from Clonetics, San Diego, CA were grown according to directions of the suppliers. Cells were grown from frozen stocks and used for 1-3 passages. Quinidine, TSA, and all trans-retinoic acid were purchased from Sigma Chemical Company (St. Louis, MO). The cell permeant proteasome inhibitors. MG-132 and lactacystin, were purchased from Calbiochem-Novabiochem (San Diego, CA).

Growth inhibition assays:

Growth inhibition by cell numbers was assayed by plating cells in 35 mm^2 dishes (1- 1.5 X 10^5) containing DMEM/ 5% FBS plus quinidine (90 uM). Viable cells were counted using a hemacytometer and trypan blue (0.02%) exclusion was used as an indicator of viability. Cell growth was also monitored in a 96-well plate format using the One Solution Cell Proliferation Assay (Promega, Madison, WI), that is based upon metabolic bioreduction of a tetrazolium compound (Owen's reagent) to a colored formazan product that absorbs light at 490 nm. The plating density for the 96-well dishes (cells/well) was varied depending upon the relative growth rates of the cell lines as follows: HMEC (2000), MCF-7 (1000), MDA-MB-231 (500), T47D (1500), and MCF-7ras (500). The One Cell Proliferation Assay Reagent was added to each well and incubated for 2 h at 37^0C. Absorbance (490 nm) was read using a Molecular Devices PC340 (Sunnyvale, CA).

Microscopic Imaging

Cells were plated (1 x 10^5) on sterile coverslips in 35 mm^2 dishes, and grown for 96 h in DMEM / 5% FBS supplemented with either 10 uM all-trans retinoic acid (in 0.01% ethanol) or 90 uM quinidine (in H_20). Control cells were grown in medium containing a final concentration of 0.01% ethanol. The presence of ethanol had no effect upon lipid droplet accumulation compared to cells grown in DMEM / 5% FBS. Cells were fixed in 3.7% formaldehyde – PBS, rinsed in PBS, then treated briefly with 0.4% Triton X-100 in PBS. After rinsing three times in PBS, the cells on coverslips were incubated for 30 min at 37^0C with a primary antibody to cytokeratin 18 (1:1 dilution, provided by Dr. Guillaume van Eys, Maastricht University), rinsed and incubated (30 min/37^0C) with Texas Red conjugated secondary antibody (goat anti-mouse IgG, Sigma). Alternatively, cells were incubated with fluorescein-phalloidin (5 ug/0.1ml, Sigma) in the dark for 40 min at room temperature, rinsed and incubated for 5 min (room temperature) with the fluorescent lipid stain, Nile Red (1:10,000 dilution of a 1mg/ml acetone solution, Sigma) [14-15]. All coverslips were rinsed in PBS, and mounted with Fluoromount –

G containing 2.5% N-propyl galate. Images were obtained using a Zeiss Axiovert 100 M confocal microscope (63 X objective).

Immunoblotting:

Cells were harvested from confluent T-75 flasks and subcultured ($1x10^6$) in $60mm^2$ dishes. On subcultivation, this confluent population of cells (85% in G1) synchronously proceeded through the cell cycle. To prepare whole cell lysates, the cells were harvested at the times indicated by scraping into ice-cold buffer (50 mM Tris-HCl, 0.25 M NaCl, 0.1% (v/v) Triton X-100, 1mM EDTA, 50 mM NaF and 0.1 mM Na_3V0_4, pH 7.4). Protease inhibitors (protease inhibitor cocktail, Roche Molecular Biochemicals, Indianapolis, IN) were added immediately. Cell lysates were centrifuged in an Eppendorf microcentrifuge (14,000 rpm, 5 min) at 4^0C, and the supernatants used in immunoblotting experiments.

Histones were prepared from cells grown at a density of 1 x 10^7/T-162 flask. To harvest the cells, the flasks were placed on ice, and the growth medium removed. Following a quick rinse with ice-cold PBS, cells were scraped into 1ml of ice-cold lysis buffer (10mM Tris HCl, 50 mM sodium bisulfite, 1% Triton X-100 (v/v), 10 mM $MgCl_2$, 8.6% sucrose, pH 6.5) and nuclei released by Dounce homogenization. The nuclei were collected by centrifugation (3,000 rpm, 10 min, SS-34 rotor) and washed three times with the lysis buffer. Histones were extracted from the crude nuclear pellets using the procedure of Nakajima et al. [16]. The pellets were resuspended in 0.1 ml of ice-cold sterile water using a vortex and concentrated H_2SO_4 to 0.4 N was added. The preparation was incubated at 4^0C for 1 h, then centrifuged (17,000 rpm, 10 min, Sorvall SS-34 rotor). The supernatant containing the extracted histones was mixed with 10 ml of acetone, and the precipitate obtained after an overnight

incubation at -20^0C, was collected and air-dried. The acid-soluble histone fraction was dissolved in 50 ul of H2O, and stored at-70^0C.

The protein concentration of the whole cell lysate supernatants or histone preparations was determined using the BCA protein assay (Pierce, Rockford, IL) and bovine serum albumin as a standard. Equal amounts of protein were loaded onto SDS - polyacrylamide gels. Molecular weights of the immunoreactive proteins were estimated based upon the relative migration with colored molecular weight protein markers (Amersham Life Science Inc., Arlington Heights, IL). Proteins were transferred to polyvinylidene difluoride membranes (PVDF) (Novex, San Diego, CA) and blocked at 4^0C using 5% nonfat milk blocking buffer (1M glycine, 1% albumin (chicken egg), 5% non-fat dry milk and 5% FBS) overnight. The membranes were incubated with primary antibodies for 3 h at room temperature. The antibody sources were as follows: mouse monoclonal anti-p27 (F-8,SC-1641), rabbit polyclonal anti-CDK4 (C-22), goat polyclonal anti-HDAC1 (N-19,SC-6299), all from Santa Cruz Biotechnology (Santa Cruz, CA), mouse monoclonal anti-pRb (14001A) from PharMingen (San Diego, CA), mouse monoclonal anti-cyclin D1 (NCL-cyclin D1, #113105) from Novocastra (Burlingame, CA), and mouse monoclonal anti-p16 (Ap-1), p21 (WAF1,Ap-1), p53 (Ap-6) from Calbiochem (Cambridge, MA) and anti-acetylated histone H4 antibody (1:500, rabbit polyclonal, Upstate Biotechnology, NY). The primary antibodies were diluted at 1:500 in Western washing solution (0.1% non-fat dry milk, 0.1% albumin (chicken egg), 1% FBS, 0.2%(v/v) Tween-20, in PBS, pH 7.3). The antigen-antibody complexes were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies (goat IgG-HRP (SC-2020), rabbit IgG-HRP (SC-2004) or mouse IgG-HRP (SC-2005) from Santa Cruz Biotechnology) at a final dilution of 1:3000 in Western washing solution. After washing three times with Tris-buffered saline (TBS: 10 mM Tris-HCl, pH 7.5, 0.5M NaCl and 0.05% (v/v) Tween 20), antibody binding was visualized using enhanced chemiluminescence (SuperSignal West Pico, Pierce) and autoradiography.

In vitro HDAC activity assay-

Quinidine-HCl was added to a chicken erythrocyte cellular extract, which contained HDAC activity, at concentrations of 90 uM [18]. HDAC assays were performed as described in Hendzel et al. [19]. Briefly, the cellular extract was incubated with 500 ug acid-soluble histones isolated from [^{3}H] acetate-labeled chicken erythrocytes for 60 min at 37^{0}C. Reactions were terminated by addition of acetic acid/HCl to a final concentration of 0.12N/0.72N. Released [^{3}H]-acetate was extracted using ethyl acetate and quantified by scintillation counting. Samples were assayed three times, and the non-enzymatic release of label was subtracted to obtain the reported values.

Results

Hyperacetylation of histone H4

Antibodies that recognize acetylated forms of histone H4 have been used as a probe for agents that cause histone hyperacetylation. [19]. In Western blot experiments, we compared the ability of quinidine to cause hyperacetylation of H4 in MCF-7 cells with that of TSA, an established HDAC inhibitor, known to inhibit proliferation in MCF-7 cells [20]. Acetylated H4 appeared rapidly, within 2 h, in MCF-7 cells exposed to quinidine (Fig. 1A). Acetylated H4 levels were most evident between 12-24 h of treatment with either quinidine (Fig. 1A) or TSA (Fig. 1B). Hyperacetylation of H4 was a transient response to both agents, and the levels of acetylated H4 were diminished by 48 h. Acetylated H4 is present in MCF-7 cells but under the conditions of Western blotting and immunochemical staining was not detected in control cells (data not shown).

HDAC1 is expressed in MCF-7 cells, and this enzyme contributes to the control of histone deacetylation rates [18]. Quinidine caused the rapid disappearance of HDAC1 from MCF-7 cells. HDAC1 protein levels in quinidine treated cells were reduced after 15-20 minutes compared with

control cells, and HDAC1 protein was undetectable between 30 minutes and 6 hours (Fig. 1E and 1F). Partial restoration of HDAC1 protein occurred beginning at 9 h of treatment, but even after 48 h, HDAC1 levels in quinidine-treated cells were still less than control cells. Levels of HDAC1 protein in control MCF-7 cells were relatively constant during this time (Fig. 1C). TSA-treated MCF-7 cells also showed reduced levels of HDAC1 protein as early as 30 minutes after drug addition, and the reduced HDAC1 protein level was maintained through 48 h (Fig. 1D). The data indicate that loss of HDAC1 protein might contribute to the H4 acetylation response to both TSA and quinidine. However, HDAC1 protein levels were never reduced by TSA below the level of detection as was observed with quinidine. In light of the more extensive H4 acetylation response to TSA than quinidine, we conclude that the direct inhibition of HDAC1 catalytic activity by TSA remains an important component of the H4 acetylation response *in vivo.* In addition, the time course of the HDAC1 response to quinidine and TSA differ. In response to quinidine, there is initially a more marked decrease in HDAC1 protein levels but a more rapid recovery. TSA treatment caused a sustained reduction in HDAC1 protein levels through 48 h.

To determine if the rapid loss of HDAC protein in the presence of quinidine were mediated through the 26S proteasome pathway, MCF-7 cells were treated simultaneously for 30 min with quinidine and MG-132 (30 uM), an inhibitor of the 26S proteasome. Cells treated with 90 uM quinidine showed a complete loss of HDAC1 protein which was prevented when MG-132 and quinidine were added simultaneously (Fig. 2A). Treatment with the solvent, DMSO, or MG-132 in solvent (0.1%) caused a modest reduction in the level of HDAC1 protein. These reductions in HDAC1 did not elicit a detectable stimulation of H4 acetylation, and we suggest that other HDAC enzymes present in MCF-7 cells, insensitive to DMSO, could compensate for the lost HDAC1 in the maintenance of deacetylated histone H4. This action of quinidine on HDAC1 protein was not reflected in a general decrease in cellular protein content [12], nor were all cell cycle regulatory proteins down-regulated in MCF-7 cells

in the presence of quinidine (e.g., p21/WAF1 and p53 protein, Fig. 3). Additional studies are required to define the spectrum of proteins affected by quinidine in a proteasome-sensitive manner. Quinidine (90 or 250 uM) did not inhibit the activity of the isolated chicken erythrocyte HDAC1 enzyme *in vitro* (data not shown) suggesting that quinidine caused histone hyperacetylation by eliciting a rapid and transient loss of HDAC1 protein without a direct inhibition of the HDAC enzyme. The suppression of HDAC protein levels in MCF-7 cells was accompanied by a decrease in HDAC enzyme activity in the cell extracts. Histone acetylation and depressed HDAC1 protein levels persisted for approximately 48 h in the presence of quinidine. When MCF-7 cells were exposed to quinidine for 24 h in the presence of either MG-132 or lactacystin, there was no detectable H4 acetylation (Fig. 2B). These results support the idea that quinidine-induced loss of HDAC1 protein is involved in the H4 acetylation response via a proteasomal-sensitive pathway.

G1 phase cell cycle regulatory profile in MCF-7 cells

G1 cell cycle arrest is characteristic of HDAC inhibitors, and reports of alterations in several cell cycle proteins in cells exposed to HDAC inhibitors, particularly, the elevation of the p21/WAF1 protein are numerous [21-23]. It was of interest to determine whether p21/WAF1 and other key cell cycle regulatory proteins such as the retinoblastoma protein (pRb) and the G1 phase cyclin-dependent kinase activator, cyclin D1 were targets of quinidine action in MCF-7 cells. Western blotting analysis showed that by 12 h, the levels of p21/WAF1 were increased in response to quinidine treatment approximately 11-fold, and this elevated level of protein expression persisted through 48 h. A small, less than 2-fold increase in p27 levels was observed in cells exposed to quinidine for 24 –48 h, while levels of p16 were unchanged (Fig. 3). Quinidine treatment decreased cyclinD1 and CDK4 protein levels by 12 h of treatment (Fig. 4), indicating that the CKI inhibitor, p21/WAF, as well as an important G1 phase target of p21/WAF1, the cyclin D1 / CDK4 complex, are early targets of quinidine

in MCF- 7 cells. This profile of activity is consistent with the observed cell cycle arrest of quinidine-treated MCF-7 cells in mid-G1 phase [12].

In MCF-7 cell extracts probed using anti-Rb antibodies, two separate but closely migrating bands were distinguishable. The upper band contained more highly phosphorylated pRb and the lower band, unphosphorylated or hypophosphorylated pRb. Control cells showed a faint pRb signal at 12 h , typical of cells in early G1 phase, and increased expression of both phosphorylated and unphosphorylated pRb at 24 and 48 h. Quinidine treated MCF-7 cells had no detectable hyperphosphorylated pRb at any time point examined, and total levels of Rb protein failed to increase with progression through G1 phase as seen in the control, proliferating cells (Fig. 3). The decrease in Rb phosphorylation level was predictable based on the increase in p21/WAF1 and decreased levels of both cyclin D1 and CDK4 (Fig. 4). In addition, Nakanishi et al. [24] showed that p21/WAF1 can bind Rb protein and block its phosphorylation. However, the actions of quinidine upon p21/WAF1 and cyclinD/CDK4 activity do not explain why the levels of total Rb protein were so low. Reductions in the cellular content of phosphorylated Rb protein in MCF-7 cells by quinidine is an important additional level of cell cycle control that effectively attenuates progression of cells out of G1 phase, and has been reported in other tumor cell lines in response to HDAC inhibition [22]. In Fig. 5 we show data suggesting that the 26S proteasome pathway regulates the total pRb content. MCF-7 cells incubated for 24 h in MG-132 or MG-132 plus quinidine had more total pRb than cells incubated with quinidine alone. Thus, quinidine promoted the loss of both HDAC1 and pRb, and inhibition of the 26 S proteasome pathway restored the levels of both of these proteins to that seen in the untreated cells. We have no direct evidence that quinidine promotes the proteasomal degradation of either protein. We hypothesize that quinidine may direct degradation of HDAC1 by the proteasome or, alternatively, quinidine might stimulate the proteasomal degradation of other regulatory factor(s) that act to maintain HDAC1 and pRb protein levels.

MCF-7 cells express wild-type p53 protein. Normal p53 is a short-lived protein that is maintained at low levels, but in response to cell stress or DNA damage, p53 is stabilized and accumulates in the nucleus where it functions as a transcription factor inducing p21, G1 cell-cycle arrest and apoptosis [25]. Wild-type p53 down-regulates pRb levels in MCF-7 cells [26]. Although Saito et al. [22] showed that p53 is not required for pRb downregulation by HDAC inhibitors in all cell lines, quinidine-treated MCF-7 cells have elevated p53 levels (5-7-fold) (Fig. 3). Thus, p53 could contribute to the maintenance of the G1 cell cycle arrest in MCF-7 by sustaining p21/WAF1 protein levels and suppressing pRb protein levels.

Growth arrest and cellular differentiation in human breast tumor cell lines

In contrast to MCF-7 cells, human breast tumor cell lines T47D, MDA-MB-231 and MDA-MB-435 express p53 proteins with distinct point mutations [27]. To test for a requirement of p53, this panel of human breast tumor cell lines was exposed to quinidine, and the effects of quinidine on cell growth were compared (Fig. 6). The data shown are viable cell numbers/well, bioreductive metabolism/well, or both. In all four cell lines growth was suppressed in a concentration-dependent fashion between 10 and 90 uM quinidine, and maximal growth inhibition was observed at ~90 uM quinidine (data not shown). These data showed that growth suppression by quinidine is a p53-independent response. It is interesting that quinidine was not overtly cytotoxic in HMEC, a line of normal human mammary cells [28].

Evidence that quinidine elicited cellular differentiation in MCF-7 human breast tumor cells in conjunction with the inhibition of cell growth was obtained using maximally effective concentrations of quinidine or retinoic acid (data not shown). Antibodies directed against cytokeratin 18 [29] were used to probe the organization of the cytoskeleton (Fig. 7). In these studies, all-trans retinoic acid (10 uM) was used to compare the differentiation response [30]. Control MCF-7 cells showed expression of

cytoplasmic cytokeratin 18 in a disorganized fashion. Cells that were treated for 96 h with retinoic acid showed an increase in the intensity of the cytokeratin 18 staining and relocalization of cytokeratin 18 throughout the nucleus as well as the cytoplasm. In contrast, cytokeratin 18 staining occurred in a highly organized pattern in MCF-7 cells treated with quinidine for 96 h and the cells adopted a shape and nuclear localization more typical of columnar epithelium.

Lipid droplets are found in the cytoplasm of normal mammary epithelium [31], and cytoplasmic lipid droplet accumulation occurs in a variety of differentiating cell systems. Induction of differentiation in human breast cancer cell lines by oncostatin M [32], the HER-2/neu kinase inhibitor, emodin [33], overexpression of c-erbB-2 [34], the vitamin D analog, 1-alpha-hyroxyvitamin D5 [35], the HDAC inhibitor, sodium butyrate [36] and retinoic acid [36] is accompanied by the accumulation of cytoplasmic lipid droplets. We utilized a fluorescent stain, Nile Red to monitor lipid droplet formation in mammary tumor cells in response to quinidine. The cells were counterstained with fluorescein-phalloidin that binds actin filaments to assay for changes in the actin cytoskeleton (Fig. 8). The distribution of actin in four human breast tumor cell lines, MCF-7, T-47D, MDA-MB-231, and MDA-MB-435 is seen clearly in the control cells. Three of these lines show strong nuclear staining of actin characteristic of transformed cells, while the fourth, MDA-MB-435, shows more cytoplasmic actin. The presence of quinidine did not alter the actin cytoskeleton as compared with control cells. Lipid droplet accumulation was weak or absent in the control cell lines, and increased by retinoic acid and quinidine. Lipid droplet accumulation was more marked in all four cell lines treated with quinidine than with retinoic acid. These data demonstrate that induction of a more differentiated phenotype is a general response of human mammary tumor cells to quinidine.

Hyperacetylation of Histone H4 in Mammary Tumor Cell Lines by Quinidine

To determine whether differentiation and histone acetylation were linked, we investigated the histone H4 acetylation status of quinidine treated T47D, MDA-MB-231 and MDA-MB-435 cells. MCF-7, MCF-7-ras, T47D, and MDA-MB-231 cells were incubated for 24 h in the presence or absence of quinidine, and then histones were extracted for immunoblotting. Fig. 9 shows that histone H4 was hyperacetylated in all cell lines treated with quinidine. Control cells contained no hyperacetylated histone H4.

Discussion

Quinidine-induced histone H4 hyperacetylation in MCF-7 human breast carcinoma cells can be attributed to the rapid elimination of HDAC1 protein, a response that was blocked by MG-132 and lactacystin, two inhibitors of proteasome-mediated proteolysis. HDAC1 protein was undetectable within 30 min after the addition of quinidine to the medium of MCF-7 cells, and hyperacetylated histone H4 appeared between 1 and 2 h. Levels of HDAC1 protein were completely suppressed between 0.5 and 6 h, and during this time H4 acetylation levels increased. H4 acetylation was maintained at 12 and 24 h, despite the partial restoration of HDAC1 protein at these same time points. These data indicate that quinidine induced reductions in HDAC1 protein levels are unlikely to fully explain the regulation of H4 acetylation state in MCF-7 cells by quinidine. Additional HDAC enzymes or effects upon histone acetylation rates could possibly play a role as well.

An earlier study showed that over this initial 48 h period, 80 % of the MCF-7 cell population had shifted into G0, a quiescent state marked by the absence of Ki67 antigen immunoreactivity [12]. Cellular differentiation manifested as the accumulation of lipid droplets and a reorganization of the cytokeratin 18 cytoskeleton was evident after this initial 48 h period. Quinidine exhibited all the responses typical of known HDAC inhibitory drugs, with the exception that quinidine had no direct

inhibitory effect upon HDAC1 enzymatic activity. We conclude from the current studies that quinidine is a novel differentiating agent that causes histone hyperacetylation, in part, by physical elimination of HDAC1 protein rather than the inhibition of HDAC enzymatic activity.

Histone H4 hyperacetylation and induction of cellular differentiation by quinidine were seen in a panel of human breast tumor cell lines that were selected for study on the basis of their diversity of genetic backgrounds. The differentiation response to quinidine was independent of the estrogen receptor (ER) status. Cell lines representative of ER-positive and ER-negative human breast carcinoma cells were induced to differentiate in the presence of quinidine. The ER status of the estrogen receptor positive cell lines is MCF-7 (ER-alpha, ER-beta), T47D (ER-alpha and ER-beta), and MDA-MB-231 (ER-beta). MDA-MB-435 cells expressed very low levels of ER-beta [37,38]. MCF-7 and T47D cells display an epithelial morphology and show similarities with mammary ductal and luminal epithelial cells, respectively [30,39]. MDA-MB-231 exhibit an elongated cellular morphology which is also typical of MDA-MB-435 cells. Our results demonstrate that quinidine is a differentiation agent in both types of mammary tumor cells.

HDAC inhibitors reverse the transformed phenotype of NIH3T3*ras* cells, and this property has been used successfully as a screening assay for the identification of new HDAC inhibitors [40,41]. Quinidine elicited a more differentiated phenotype in MCF-7*ras* cells, an MCF-7 cell derivative produced by stable transformation with v-Ha-*ras,* thus demonstrating that quinidine, like other HDAC inhibitors, can reverse an H-*ras* induced phenotype.

Quinidine induced differentiation independently of wild-type p53. The ability of quinidine to cause differentiation of p53 mutant cell lines is consistent for a role of histone hyperacetylation in the response. HDAC inhibitors typically induce a p53-independent activation of p21/WAF1 gene expression [5,22]. Growing MCF-7 and T47D cells express p21/WAF1 protein in moderate to low levels [42], and quinidine raised p21/WAF1 protein levels in MCF-7 cells approximately 11-fold

within 12 h. Although p21/WAF1 was reported to be low to undetectable in MDA-MB-231, p21/WAF1 was detected in Western analyses of both MDA-MB-231 and T47D cells in a p53-independent fashion in response to serum deprivation, adriamycin etoposide [42,43], and quinidine (data not shown). These data support the idea that the p21/WAF1 gene is present, but inactive in growing MDA-MB-231 cells. Since histone hyperacetylation of the p21/WAF1 gene occurs in response to HDAC inhibitors, it might be involved in the pathway of p53-independent activation of p21/WAF1 gene expression [5].

The processes of cellular differentiation and cell cycle progression are interdependent. G1 arrest is a necessary but insufficient condition for differentiation in numerous cell types including leukemic cells, keratinocytes, colonic epithelium, and muscle cells. In all of these cells, induction of p21/WAF1 protein and G1 cell cycle arrest occurred prior to differentiation [44-50], and was generally independent of p53. We hypothesize that the differentiated state can be viewed as a cellular response to G1 arrest, requiring a change in gene expression profile and suppression of cell death pathways. The response of MCF-7 breast tumor cells to quinidine is consistent with this model.

To begin to understand how quinidine might elicit G1 arrest of MCF-7 cells, we have focused on the action of quinidine as a potassium channel blocking agent. Quinidine enters cells and inhibits cardiac potassium channels by binding to the intracellular face of the ion pore [51]. Although the location of the quinidine binding site on the ATP-sensitive potassium channel is unknown, quinidine is freely permeable across membranes and inhibits the ATP-sensitive potassium channels whether it is applied to the external or internal surface of a lipid membrane bilayer [52].

In the presence of quinidine, MCF-7 cells accumulate at a position 12 h into G1 phase [12]. This position, defined by cell cycle arrest and release experiments, precedes the lovastatin arrest point by 5-6 h, and is clearly distinct from the restriction point described by Pardee [53] near the G1/S transition. The present work showed that quinidine treatment caused elevated levels of p53 and p21/WAF1

protein by 12 h (Fig. 3), the point within G1 where MCF-7 cells arrest in response to quinidine [12]. When p53 and p21/WAF1 proteins were assayed before 12 h, p53 was undetectable, and p21 was first detected after 8 h of quinidine treatment (data not shown), suggesting a p53-independent induction of p21/WAF1 occurred prior to arrest in G1. Cdk4 and cyclin D1 protein levels were also reduced, as was cdk4 activity as demonstrated by the abundance of hypophosphorylated Rb protein. Based upon our observations in MCF-7 cells, we conclude that p21/WAF1 protein levels become elevated prior to the G1 arrest in response to quinidine, and could initiate the G1 arrest. Hypophosphorylated Rb protein is prominent in quinidine-treated MCF-7 cells and this could act to sustain the G1 state by preventing the transition into S phase. The G1 arrest induced by quinidine in MCF-7 cells was correlated with the blockade of ATP-sensitive potassium channels in MCF-7 cells [12,54,55]. Direct evidence for the involvement of potassium ions in the G1 arrest was provided using, valinomycin a potassium-selective ionophore to stimulate a G1 - S phase transition in the presence of quinidine [12].

In summary, quinidine, a drug that is used therapeutically in the treatment of malarial infections and cardiac arrhythmia, was shown to be useful as a chemical probe for unveiling a novel mechanism of cellular differentiation in human breast tumor epithelial cells. Quinidine caused histone H4 hyperacetylation and cellular differentiation in human breast tumor cells following the rapid loss of HDAC1 involving a proteasome-dependent pathway. We argue that the action of quinidine upon ATP-sensitive potassium channels in MCF-7 cells provides a rational basis for understanding the molecular events underlying the differentiation response.

Fig. Legends

Fig. 1. Histone Hyperacetylation in MCF-7 Cells. A) Histones were extracted from cells grown in the presence of 90 uM quinidine for 0.5 , 1, 2, 6, 12 , or 24 h; histones (20 ug/lane) were electrophoresed in 15 %polyacrylamide gels containing 1% SDS, and assayed for the presence of acetylated H4 by immunoblotting. B) Histones were extracted from cells grown in the presence of 300 nM TSA for 0.5, 6, 12, 24 or 48 h; 20 ug of histone/lane were electrophoresed and analysed for acetylated histone H4 by immunoblotting. C,D) HDAC1 protein in whole cell lysates were prepared from control MCF-7 cells at 0.5, 9, 12, 24 or 48 h (C) or cells treated with 300 nM TSA 0.5, 12, 24 or 48 h (D); 50 ug protein/lane were electrophoresed in 12% polyacrylamide gels containing 1% SDS, and assayed for HDAC1 protein by immunoblotting. E,F) HDAC1 protein in whole cell extracts from cells grown in the presence of 90 uM quinidine for E)15,20, 30 min or 9,12, 24 or 48 h or F) 0.5,1,2, or 6 h; extracts were electrophoresed (50 ug protein/lane) and assayed for HDAC1 protein by immunoblotting.

Fig. 2. Protection of HDAC1 protein by proteasome inhibitors. A) MCF-7 cells were released from confluency and subcultured in normal growth medium supplemented with 30 uM MG-132 in 0.1% DMSO, 90 uM quinidine plus 0.1%DMSO or 0.1% DMSO alone as indicated. Cells were harvested after 30 min, and Western blot analysis of HDAC1 protein was performed as detailed in methods. B) MCF-7 cells were cultured as described above, or with lactacystin (3 uM in 0.1% DMSO) except were harvested after 24 h, and analyzed for the presence of immunoreactive acetylated histone H4.

Fig. 3. G1 cell cycle proteins in MCF-7 cells. Cells released from confluency were plated into control medium or medium containing 90 uM quinidine. Whole cell lysates were prepared 12, 24 or 48

h after plating and assayed by immunoblotting for the CKIs , p21/WAF1 (n=3), p27 (n=1) , p16 (n=3) and p53 (n =3) after electrophoresis of 50 ug protein/lane through 12% SDS-polyacrylamide gels. Rb protein was immunoprecipitated from 500 ug of whole cell lysate protein using an antibody that recognizes phosphorylated and non-phosphorylated pRb [56]. This entire immunoprecipitate was electrophoresed in a 7.5% SDS-polyacrylamide gel and immunoblotted using this same antibody. Results shown are typical of two independent analyses.

Fig. 4. Cyclin D – CDK4 in MCF-7 cells. Confluent MCF-7 cells were subcultured in control medium or medium containing 90 uM quinidine. Whole cell lysates were prepared 0.5, 12, 24 and 48 h after subculture. Equal protein aliquots (50ug/lane) were electrophoresed in 12 % SDS-polyacrylamide gels, and assayed for cyclin D1 and CDK protein levels by immunoblotting. Results shown are representative of three independent experiments.

Fig. 5. Proteasome inhibitor modulates retinoblastoma protein levels. Confluent MCF-7 cells were subcultured in the presence of 90 uM quinidine, 30 uM MG-132, or quinidine + MG-132 for 24 h, then harvested, and whole cell extracts (100ug/lane) were analyzed for pRb. A Coomassie blue stained protein is shown as the loading control.

Fig. 6. Growth of human breast cell lines in quinidine. MCF-7, MDA-MB-231, T47D, MCF-7ras tumor cells and normal HMEC were replica plated in 96-well plates in control medium (open symbols) or medium containing 90 uM quinidine (solid symbols). Cell growth as monitored using the MTS assay is shown with solid lines. Results shown are the average of quadruplicates in one experiment. Quinidine had no effect on MTS metabolism in the MCF-7ras cell line (data not shown). Viable cell counts/dish of replica plated MCF-7, MDA-MB-231, T47D and MCF-7ras tumor cells and normal

HMEC cells in 35 mm^2 dishes in control medium (open symbols) and medium containing 90 uM quinidine (solid symbols) is indicated with the dashed lines. The MTS data are the result of single experiments conducted in quadruplicate for all the cell lines. The cell number (growth curve data) represent the mean and S.E. of three independent experiments for the MCF-7, T47D and MDA-231 cell lines performed in duplicate dishes (MCF-7, T47D) or single dishes (MDA-231) for each experiment. MCF-7ras data are the mean (± range) of two experiments performed in duplicate dishes. HMEC data are from one experiment performed in single dishes.

Fig. 7. Cytokeratin 18 in MCF-7 cells. Cells were replica plated (2 x 10^5) on sterile coverslips in 35 mm^2 dishes in medium containing 0.01% ethanol (control), 10 uM retinoic acid , or 90 uM quinidine and grown for 96 hs. Cytokeratin 18 detection using a Texas red tagged secondary antibody is shown using confocal microscopy. Data shown are typical fields representative of two independent experiments.

Fig. 8. Lipid accumulation as an index of cellular differentiation in human breast tumor cell lines. MCF-7, T47D, MDA-MB-231 and MDA-MB-435 cells were replica plated (0.8-3 x 10^5) on sterile coverslips in 35 mm^2 dishes in medium containing 0.01% ethanol (control), 10 uM retinoic acid (0.01% ethanol) or 90 uM quinidine (in water). Cells were fixed, permeabilized and then incubated sequentially with fluorescein-phalloidin to identify actin filaments and Nile Red to identify lipid droplets after 96 h. Images were obtained by confocal microscopy. The results are typical of three experiments conducted in each cell line.

Fig. 9. Histone H4 hyperacetylation in human breast tumor cell lines. MCF-7, MCF-7ras, T47D, MDA-MB-231 and MDA-MB-435 tumor cells were replica plated (1 x 10^7/T-162 flask) in control

medium (C) or medium containing 90 uM quinidine (Q) . Histones were extracted from the cells after 24 h, and 20ug/lane of histone proteins were electrophoresed in 15% SDS-polyacrylamide gels. Immunoblotting was performed to detect acetylated histone H4.

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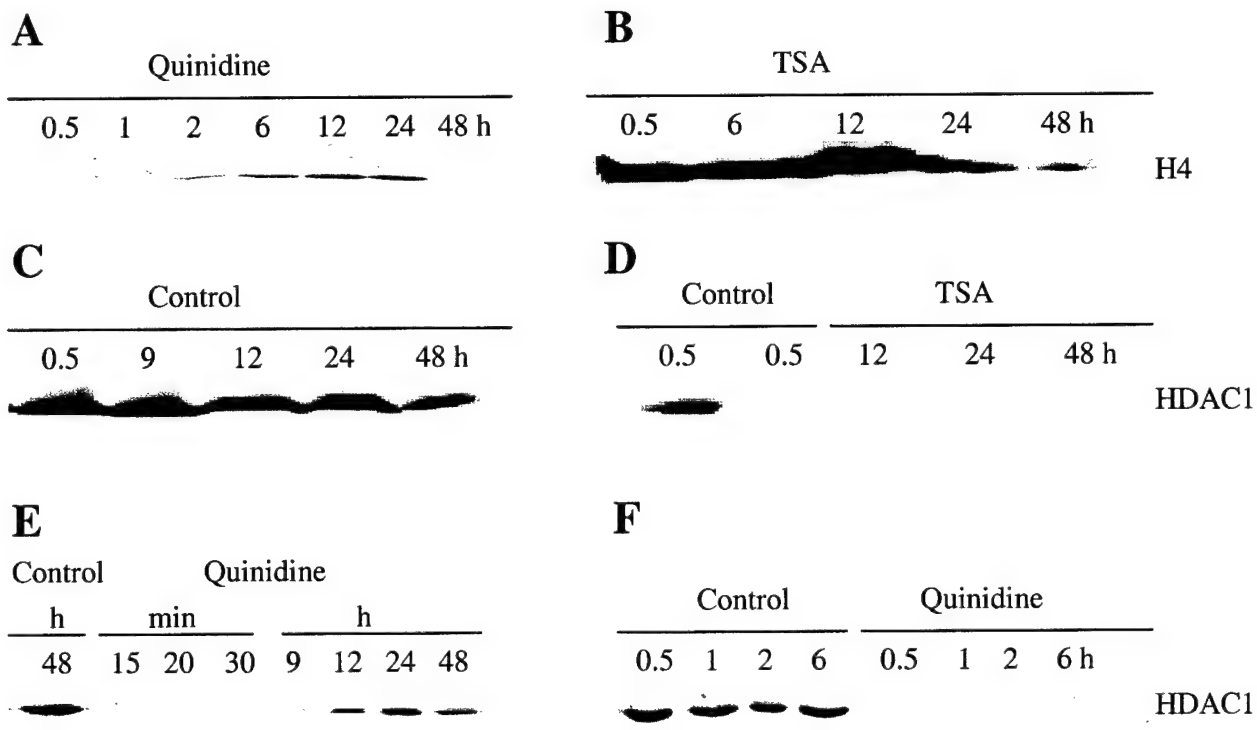
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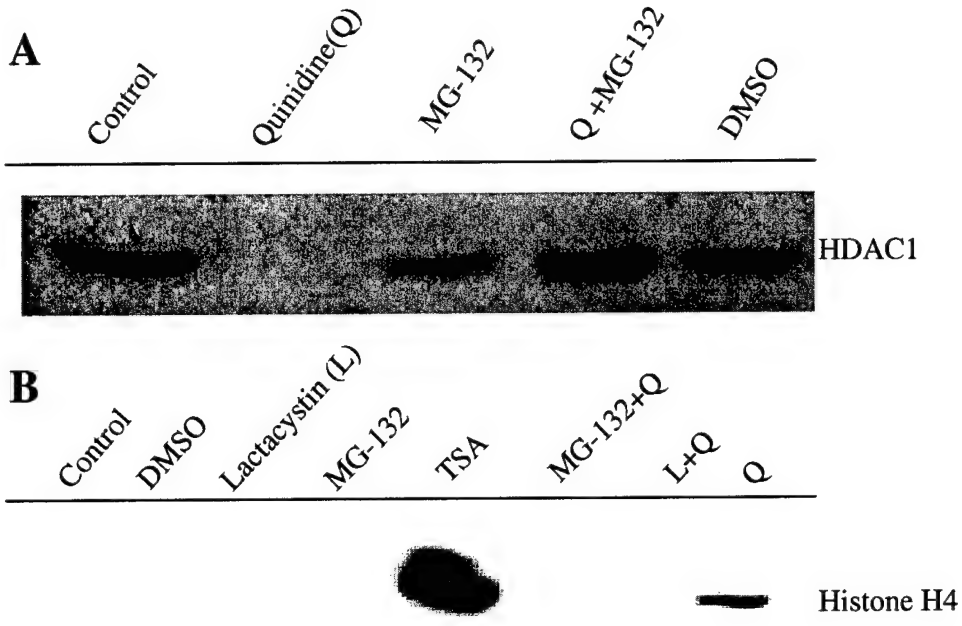
Figure 1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Quinidine(Q)
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Q +MG-132
DMSO
HDAC1
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Q
Histone H4

Figure 2

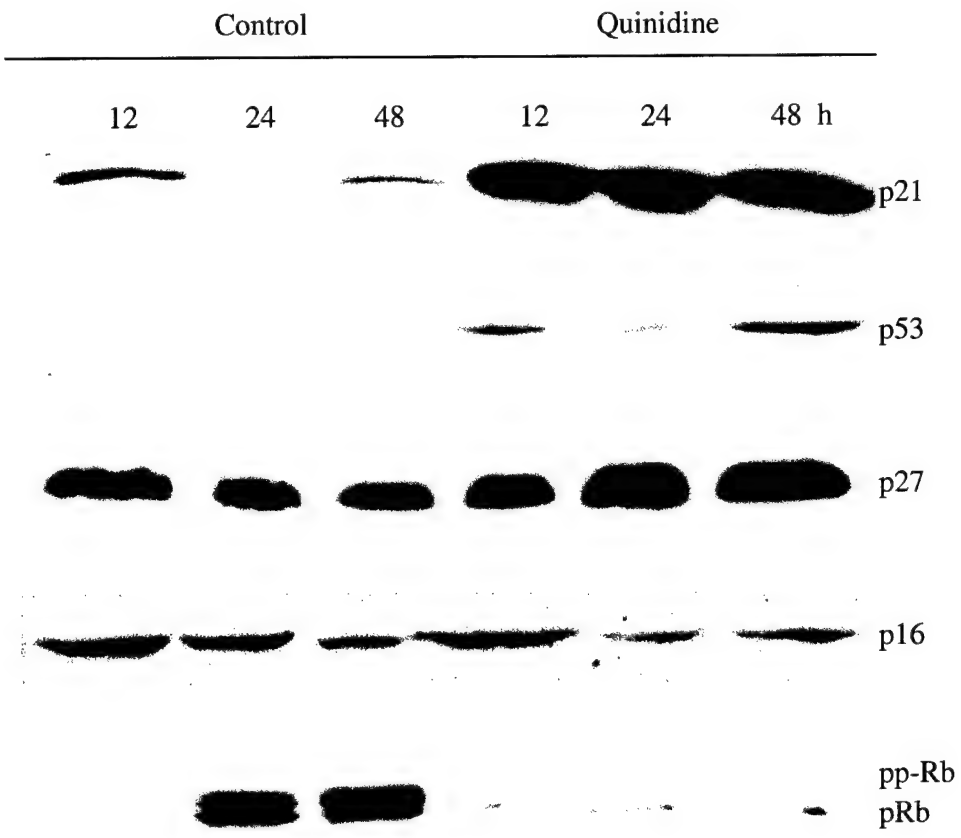


Figure 3

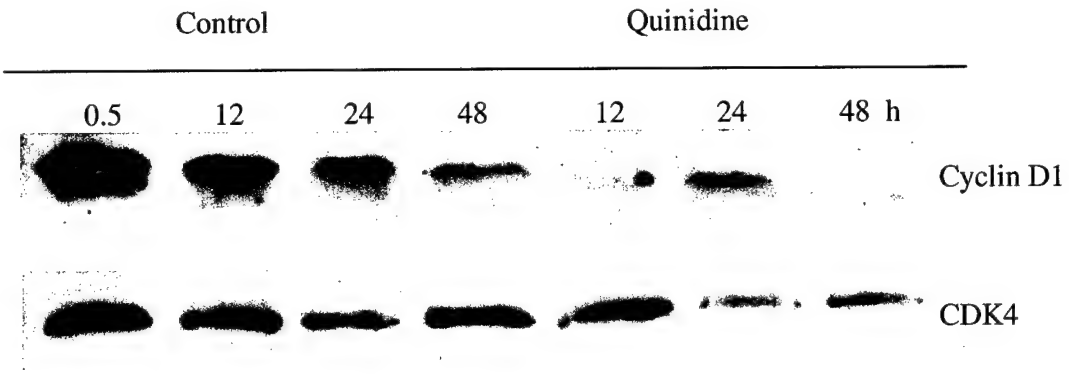


Figure 4

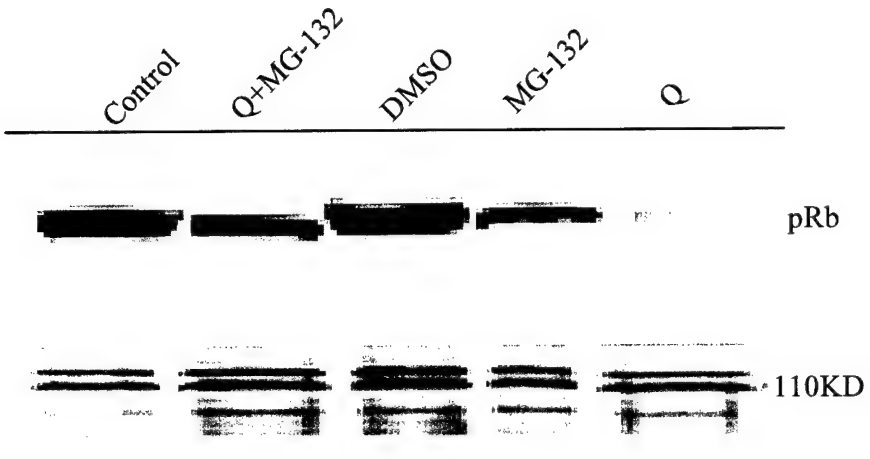
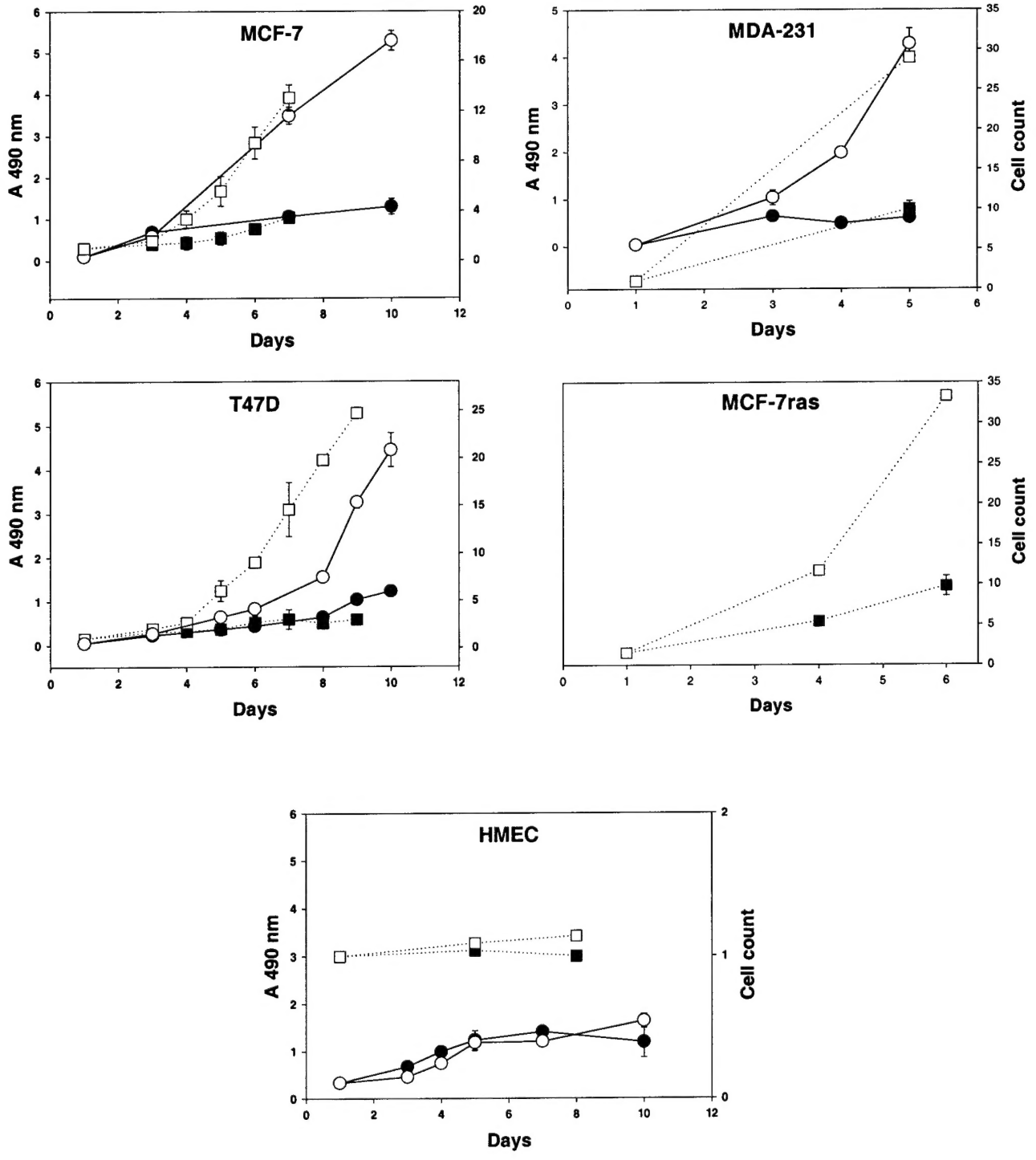


Figure 5


MCF-7
A 490 nm
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T47D
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MCF-7ras
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HMEC
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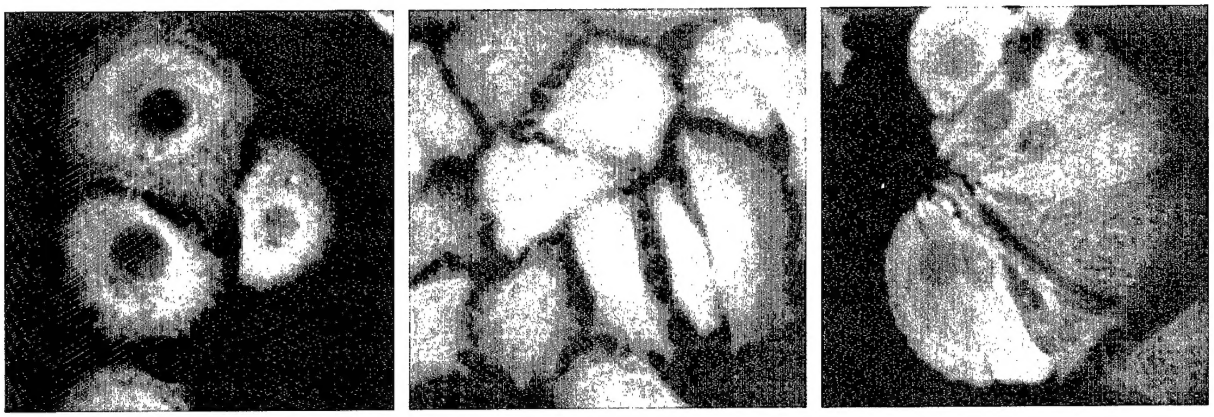
Figure 6

MCF-7

Cytokeratin 18



Control **Retinoic Acid** **Quinidine**

Figure 7

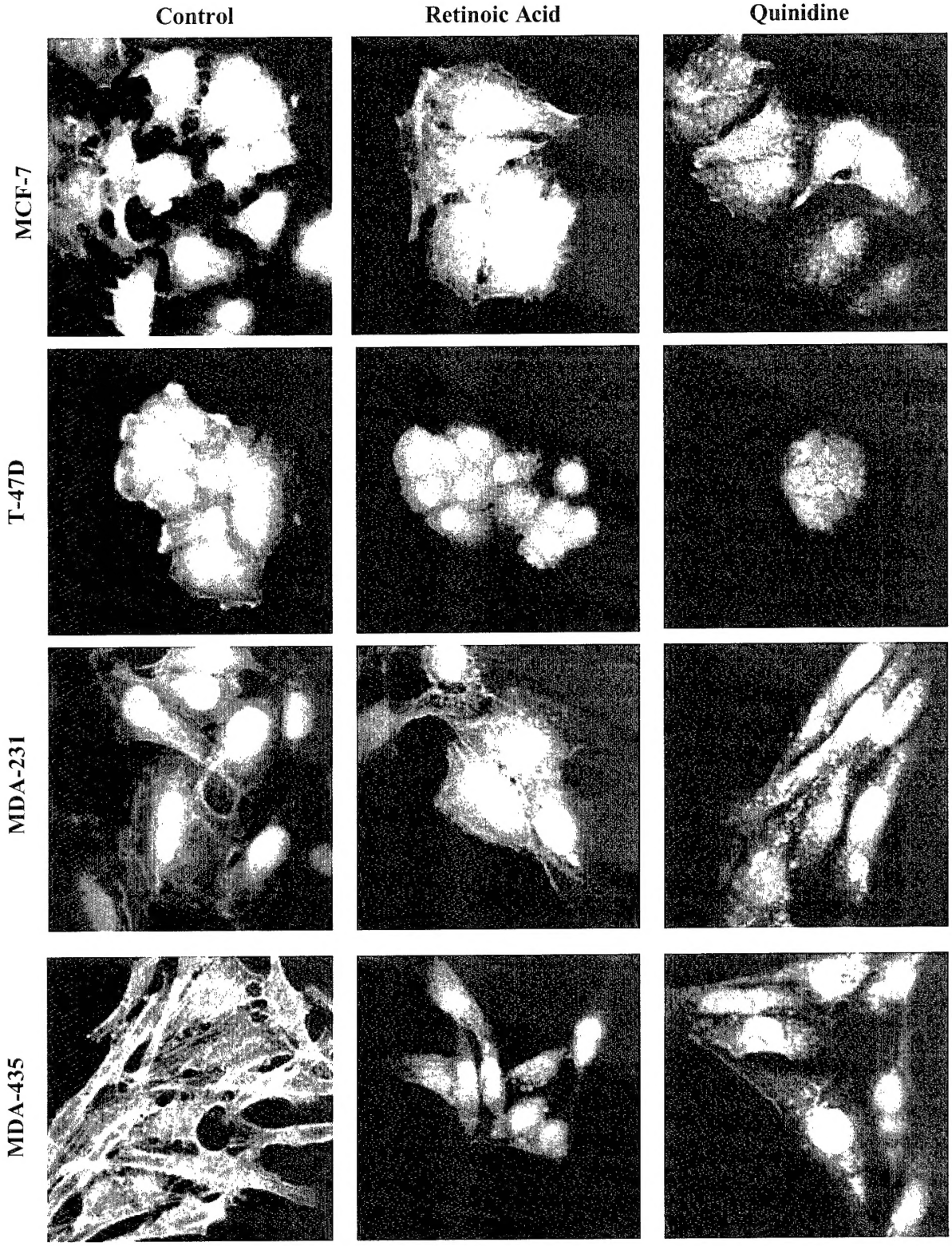


Figure 8

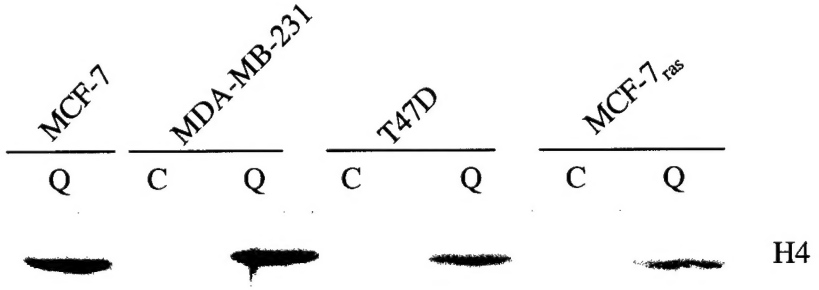


Figure 9